Expression and Functional Characterisation of Variola and Monkeypox Virus Tumour Necrosis Factor Receptor (TNFR) Proteins

Sarah Sherwood

PhD

Institute for Infection, Immunity and Innovation

The University of Technology, Sydney, Australia

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ithreeinstitute

UNIVERSITY OF TECHNOLOGY SYDNEY

CERTIFICATE OF AUTHORSHIP

I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirement for a degree except as fully acknowledged within the text.

I also certify that this thesis has been written by me. Any help that I have received in my research work and in preparation of this thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

Sarah Sherwood

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PRESENTATIONS

11th Annual Cellular Biology Meeting. Pokolbin NSW, Mar 22-25 2011

Gale A, **Sherwood S,** Johnson M, Sedger LM

Visualisation of viral and cellular tumour necrosis factor receptors (TNFRs) with mutations in the pre-ligand assembly domain (PLAD) required for viral:cellular TNFR co-localization. (Poster Presentation)

ASM 2010 Annual Scientific Meeting. Sydney NSW, July 4-8 2010

Sherwood S, Roslan N, Kerr P, Sedger LM

Defining Roles for TNFα-inhibition and TNFR-association for the Human Tropic Poxviruses Variola Virus (Smallpox) and Monkeypox Viral TNFR-like Proteins. (Oral Presentation)

Australian Virology Society Meeting. Lorne Vic, Dec 13-17 2009

Sherwood S and Sedger LM

Expression and characterisation of variola and monkeypox TNFR-like T2 proteins for potential use as TNFR based anti-inflammatory agents. (Poster Presentation)

25th Annual Scientific Research Meeting. Sydney NSW Nov 18-19 2008

Sherwood S and Sedger LM

Inhibiting Tumor Necrosis Factor Receptor Mediated Inflammation. (Oral Presentation)

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LIST OF ABBREVIATIONS

Abbreviation	**Full name**
ActD	Actinomycin D
ANT	Adenine nucleotide translocator
Apaf-1	Apoptotic protease avtivity factor 1
APRIL	A proliferating-inducing ligand
ASFV	African swine virus
BAFF	B cell activating factor
BCIP	5-bromo-4-chloro-3-indolyphophate
BID	Bcl-2 interacting protein
bp	Base pair
BSA	Bovine serum albumin
CDC	Centers for Disease Control and Prevention
cDNA	Complementary DNA
CFP	Cyan fluorscent protein
CRD	Cysteine-rich domain
Crm	Cytokine response modifier
C-terminal	Carboxy-terminal
DD	Death domain
ddH_2O	Double-distilled water
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphates
DOC	Sodium deoxycholate
dsDNA	Double stranded DNA
DTT	Dithiolthreitol
EBV	Epstein-barr virus
EDTA	Ethylene diamine tetra acitic acid
Etk	Endothelial/epithelial tyrosine kinase

EV	Enveloped virion
FADD	Fas associated protein with death domain
FAN	Factor associated with neutral shingomyelinase
FBS	Foetal bovine serum
FRET	Fluorescence resonance energy transfer
g	Centrigual force (gravity)
GITR	Glucocorticoid-induced TNF-receptor related protein ligand
HEK	Human embryonic kidney
HIV	Human immunodeficiency virus
HVEM	Herpes virus entry mediator
IFN	Interferon
Ig	Immunoglobulin
IKK	IκB kinase
IL	Interleukin
IRAK	Interleukin 1 receptor-associated kinase
IV	Immature virion
JNK	c-Jun N-terminal kinase
Kb	Kilobases
kDa	Kilodaltons
KSHV	Kaposi-s sarcoma-associated herpes virus
LB	Luria Broth
LPS	Lipopolysaccharide
M	Molar
MADD	MAPK ackivating death domain
MAPK	Mitogen activated protein kinase
MHC	Major Histocompatibility Complex
MPV	Monkeypox virus
mRNA	Messenger RNA
MTT	3-(4,5 dimthylthiazolyl-2)-2,5-diphenyl tetrazolium
MV	Mature virion
MyD88	Myeloid differentiation primary-response protein-88

Myx	Myxoma virus
NA3A	Non capsid protein 3A
NBT	Nitro blue tetrazolium
NEMO	NFκB essential modulator
NFκB	Nuclear factor κB
ng	Nanograms
nm	Nanometre
N-terminal	Amino-terminal
^{o}C	Degrees Celsius
OGTR	Office of the Gene Technology Regulator
orf	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PAMPs	Pathogen associated molecular patterns
PBR	Poxvirus Bioinformatics Resource Center
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PLAD	Pre-ligand association domain
PTPC	Permeability transition pore complex
RA	Rheumatoid arthritis
RANK	Receptor activator nuclear factor κB
RIP	Receptor-interacting kinase
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS	Sodium dodeyl sulphate
SECRET	Smallpox virus-encoded chemokines receptor
SODD	Silencer of death domain
TAB	TAK1 binding protein
TACE	TNFα converting enzyme
TACI	Transmembrane activator of CAML interactor
TAK1	Growth factor-β-activated kinase 1

TBE	Tris borate buffer with EDTA
TIRs	Terminal inverted repeats
TLR	Toll-like receptor
TNFR	TNF receptor
TNFR-SF	TNFR superfamily
TNF-SF	TNF-superfamily
TNFα	Tumour necrosis factor-α
TRADD	TNF receptor associating death domain
TRAF	TNF receptor-associated factor
TRAIL	TNFα-related apoptosis-inducing ligand
TWEAK	TNFα-related weak inducer of apoptosis
VAC	Vaccinia virus
VAR	Variola virus
VDAC	Voltage dependent anion channel
VEGI	Vascular endothelial cell-growth inhibitor
Vpr	Viral protein R
WHO	World Health Organisation
YFP	Yellow fluorscent protein

ABSTRACT

Tumour necrosis factor-α (TNFα) is a pleiotropic cytokine that plays a critical role in cellular response to virus infection. Virtually all poxviruses encode genes that are homologous to human tumour necrosis factor receptors (TNFRs). The viral "T2" TNFR proteins are well characterized from *Leporipox* viruses, myxoma (Myx) and shope fibroma virus. MyxT2 has previously been shown to bind to and inhibit rabbit TNFα in a species-specific manner and, more recently, has been shown to bind to human cellular TNFRs and inhibit TNFR1-induced cell death in a non species-specific manner. In contrast, the human-tropic *Orthopoxviruses* TNFR proteins have been poorly characterised, since variola virus (VAR) existed before molecular virology capabilities and the monkeypox virus (MPV) is restricted for research. This study sought to characterize the TNFR proteins, VARG4R and MPVJ2R, encoded by strict species-specific variola virus and the broad host range monkeypox virus, and compare them to the well characterized MyxT2 protein.

With WHO Smallpox Committee approval, codon optimised cDNAs for VARG4R and MPVJ2R were constructed and these proteins were expressed as C-terminal myc-His-tagged fusion proteins by transient transfection in HEK293T cells. Both VARG4R and MPVJ2R are expressed and detectable in cell lysates and culture supernatants, exactly as occurs for MyxT2. However, while MyxT2 is both a dimer and a monomer, VARG4R is predominantly a dimer and MPVJ2R is exclusively a monomer. Secreted VARG4R and MPVJ2R are heavily glycosylated consistent with their numerous N-linked glycosylation sites. In TNFα neutralization L929 cytotoxicity assays, VARG4R inhibits rabbit, mouse, human and rhesus macaque TNFα. Although monkeypox virus has an extremely broad host range, surprisingly MPVJ2R has no TNFα inhibitory activity against rabbit, human, or rhesus macaque TNFα. Consistent with MyxT2, VARG4R and MPVJ2R inhibit TNFR1-induced cell death. It has previously been demonstrated that the viral pre-ligand association domain (PLAD) is essential for MyxT2 inhibition of TNFR1-induced cell death. Interestingly, MyxT2 PLAD, VARG4R PLAD and MPVJ2R

PLAD proteins also inhibit TNFR1-induced cell death, confirming the critical role of the viral PLAD domain in the function of these viral TNFR proteins.

Collectively these data suggest that, like MyxT2, secreted VARG4R protein acts as a functional TNFα-inhibitory factor during variola infection, but poxviruses with a broad host range, such as monkeypox, use other non species-specific mechanisms for host immune evasion. Overall, this study expands on our limited knowledge of variola and monkeypox viruses' mechanisms of immune evasion and further confirms the pivotal role of the viral PLAD in viral inhibition of TNFα-TNFR signaling.